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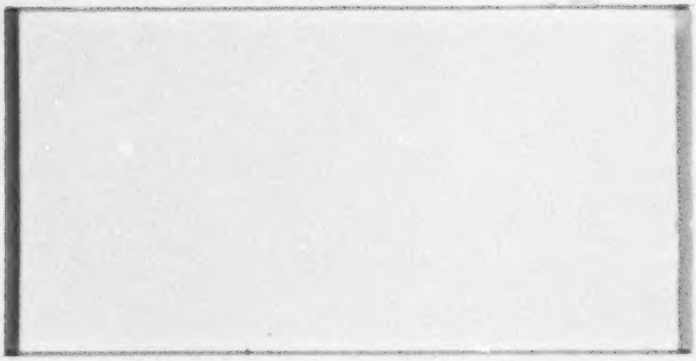

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TRITIUM OXIDE UPTAKE AND DESORPTION KINETICS IN A PRIMARY PRODUCER: *CHLORELLA PYRENOIDOSA*

Report No 83-383-K

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ABSTRACT

The alga, *Chlorella pyrenoidosa*, grown in batch culture under chronic tritium oxide exposure was used to model behavior of tritium at the primary producer level of an aquatic food chain. The specific activity ratio of organically bound tritium to medium tritium increased during initial growth stages, then reached an asymptotic steady state value of 0.59 after approximately seven cell doublings. The intracellular to extracellular concentrations of tritium oxide appeared to be in equilibrium. Loss of previously formed organically bound tritium in cells transferred to tritium-free media averaged less than 5% for exponential growth phase cultures which had undergone more than three cell doublings. Over a comparable time period, a greater loss of organically bound tritium from stationary cells (average 13.4%) was attributed to increased degradative metabolism in senescent cultures. Concentration of tritium in organically bound form may exceed environmental tritium oxide levels under dynamic conditions in which a pulse of tritium oxide to the environment is dissipated over time.

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EXECUTIVE SUMMARY

TRITIUM OXIDE UPTAKE AND DESORPTION KINETICS IN A PRIMARY PRODUCER: *CHLORELLA PYRENOIDOSA*

T.G. Dunstall

Tritium released from CANDU nuclear reactors enters the hydrosphere as tritium oxide. Subsequent incorporation into flora and fauna may follow the same physiological processes as occur for normal water. Among the flora, photosynthesis may lead to the direct incorporation of tritium into organic matter, which may then be transferred through consumer levels of a food chain. The radiological significance of tritium within the environment is therefore dependent on the nature of its incorporation into biota, particularly with respect to potential bioconcentration or biomagnification of tritium. Although there have been a considerable number of studies pertaining to the behavior of tritium in biota, few have involved identification of the relationship under conditions of chronic (steady state) tritium exposure.

As part of the Biological Tritium Program, laboratory studies have been initiated to model tritium relationships within an aquatic food chain consisting of a primary producer (an alga), a primary consumer (crayfish), and a secondary consumer (fish). This report describes the uptake and desorption kinetics of tritium in the unicellular green alga, *Chlorella pyrenoidosa*.

Batch cultures of *C. pyrenoidosa* were chronically exposed to tritium oxide at a concentration of approximately 1 mCi/L. Tritium relationships in the alga were monitored from early (exponential) culture growth to stationary (senescent) development. The rate of tritium incorporation into the organically bound fraction of the algal cells was rapid during early culture growth, but then gradually declined and approached zero after about seven cell doublings. The asymptotic (steady state) specific activity ratio of organically bound tritium to medium

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tritium was estimated to be 0.59 (95% confidence intervals; 0.56, 0.62) indicating discrimination against tritium, relative to hydrogen, in biosynthetic reactions. This was in agreement with maximum specific activity ratios of approximately 0.5 and 0.7 previously reported for *C. pyrenoidosa* and to the ratios of 0.5 to 0.8 reported for other freshwater and marine algal species. There is no evidence to suggest that the concentration of tritium in algal organic matter exceeds tritium oxide levels, even under chronic exposure conditions.

The intracellular concentration of tritium in the tissue-free water component of the algal cells was compared to tritium oxide concentration in the medium. Analyses of algal material (packed cell volumes > 3 mL) indicated that the specific activity ratio of tissue-free water tritium to medium tritium was close to unity (range 0.99 to 1.18). This result was consistent with previous literature reports suggesting the free diffusion of tritium oxide across algal cell membranes.

Loss of tritium from organic matter was followed in cells initially grown in a tritiated medium and then transferred to tritium-free media. A small but significant loss (<5%) of organically bound tritium resulting from catabolic metabolism and/or hydrogen-tritium exchange reactions occurred among actively growing cells which had undergone more than three cell doublings. The rate of tritium loss was higher at about 13% over a comparable period of time for cells in stationary growth phase, a probable result of increased degradative metabolism. The persistence of tritium in organic matter indicates that the organically bound tritium to medium tritium ratio may exceed unity under conditions in which a pulse of tritium oxide to the environment is dissipated over time. Care should be taken not to misinterpret environmental data where specific activity ratios in biota exceed unity as evidence of bioconcentration or biomagnification of tritium in the biosphere.

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To Mr. F.J. Kee
Director of Research

TRITIUM OXIDE UPTAKE AND DESORPTION KINETICS IN A PRIMARY PRODUCER: *CHLORELLA PYRENOIDOSA*

T.G. Dunstall

1.0 INTRODUCTION

Tritium, an isotope of hydrogen, is the major radionuclide released to the environment by CANDU nuclear power reactors. Neutron activation of heavy water (D_2O) used in the moderator cooling systems of CANDU reactors is the primary source of tritium. Chronic low level releases of tritium oxide (HTO) to the aquatic environment, and to the atmosphere in vapour form, may result in the incorporation of tritium into organisms following the same physical and physiological processes as normal water. Among primary producers the reactions of photosynthesis lead to the direct incorporation of tritium into the organically bound fraction.

Previous studies on both unicellular and filamentous algae have shown the incorporation of tritiated water into the tissue-free component to be rapid (minutes) with the intra- to extracellular HTO ratio approaching unity (Kirchmann, *et al.*, 1979; Rosenthal and Stewart 1973; Harrison and Koranda 1973). Incorporation of tritium into the organically bound fraction occurred at a much slower rate (hours to days) with the ratio of tritium in the combustion water of dried tissue to that of the medium generally being in the range of 0.45 to 0.80 (Ibid; Weinberger and Porter 1953; Kanazawa *et al.* 1972; Strack 1978). Specific activity relationships of organically bound tritium (OBT) to medium tritium of less than one have been interpreted as resulting from the discrimination against tritium (compared to hydrogen) in metabolic reactions. The ratio of tritium to protium incorporation into various biochemical compounds was found to vary between 0.45 and 1.00, with highest values occurring among intermediates of the tricarboxylic acid cycle and related amino acids. These differences were explained on the basis of specific isotopic effects on different enzyme mediated reactions (Kanazawa *et al.* 1972; Rambeck and Bassham 1973).

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With the exception of the study by Strack (1978), whose experiments were run under static conditions, previous work has involved a single injection of tritium into the system following which the tritium content of the medium could be expected to decline at a rate dependent not only on cellular incorporation, but also on evaporation or the addition of tritium-free medium (Harrison and Koranda 1973; Strack *et al.* 1979). Because of the lag which exists in the incorporation of tritium into newly synthesized organic matter, the OBT to medium-T relationship has not been adequately examined under equilibrium or steady-state conditions of exposure.

In one study (Strack *et al.* 1979) involving a single loading of HTO to a continuous algal culture, the ratio OBT/medium-T was initially less than one because of the gradual replacement of unlabelled organic material with newly synthesized (tritiated) material. With continual replacement of medium (and algal cells) the tritium level of organic matter increased while medium-T declined such that a specific activity of unity was attained in the third day of the experiment. After this point the specific activity of the organic matter exceeded that of the medium, attaining a ratio of 6:1 by the end of the experiment. Discrimination due to isotopic effects was estimated at 0.8 (ie, a 4:5 ratio in the uptake of T relative to H).

The importance of a high OBT to medium-T relationship lies in the transfer of tritium through food chains and the potential for bioconcentration of tritium into organisms of higher trophic levels. As part of the Biological Tritium Program, laboratory simulation studies are being used to model tritium behavior within three trophic levels, representative of an aquatic food chain. The unicellular alga, *Chlorella pyrenoidosa*, described in this report, was considered to represent an aquatic primary producer. Tritium relationships in a primary consumer grown in tritiated water and/or fed a tritiated food source has also been investigated using crayfish as a model organism (Ontario Hydro Research Division Report in draft). Similar studies are underway for a secondary consumer (fish).

The purpose of the present study was to examine the rate of tritium incorporation and specific activity relationships (OBT to medium-T) in the unicellular alga, *Chlorella pyrenoidosa*, cultured under steady-state (chronic medium-T) conditions. Also investigated was the rate of tritium loss from the organically bound fraction following removal of tritium from the culture medium. Prior to commencement of these studies, however, it was necessary to characterize culture conditions, identify relationships between growth parameters and to develop analytical procedures to be used in determining tritium relationships in algae.

2.0 MATERIALS AND METHODS

2.1 Maintenance Cultures

Chlorella pyrenoidosa Chick (Emerson strain, culture #395) was obtained from the Culture Collection of Algae, University of Texas. Stock cultures of the algae were maintained on protease agar slants, being transferred at one to two month intervals. Following inoculation, stock cultures were grown for several days at 25°C and were then maintained at 4°C. These stocks were used to inoculate triplicate liquid maintenance cultures every two to three weeks in 50 mL Beijerinck's medium. After 7 to 10 days incubation, cultures had reached late exponential growth phase (determined by optical density) and were ready to use as the inoculum for experimental batch cultures.

2.2 Batch Cultures

Experimental cultures were established in twelve round bottom Pyrex flasks each containing 5 L Beijerinck's medium, with an initial algal density of approximately of 10^4 cells/mL). Tritium oxide (HTO) was added to each flask and triplicate 0.5 mL samples (initially 1.0 mL samples used) were removed for liquid scintillation counting of initial tritium activity. Average concentration of tritium in the cultures was 0.94 ±0.05 mCi/L. Flasks were suspended in a water bath that was maintained at a temperature of 25°C ± 1°C. A fluorescent light bank (3 x 4 W) provided each flask with approximately 90 uE/s/m^2 on a continuous basis (Figure 1). A 5% CO_2-air mixture was delivered to the cultures at the rate of approximately 8 L/h/flask to provide a carbon source for growth and to maintain cells in suspension. Prior to delivery to the flasks the CO_2-air mixture was passed through a water wash (pH 8.5) and a cotton filter to remove possible particulates. The gas wash contained the same concentration of tritium as the culture media to ensure the maintenance of steady-state tritium (HTO) levels. Exhaust air from each flask was bubbled through a non-tritium water wash and subsequently vented from the room.

Non-tritiated control cultures were grown under the same incubation conditions, with the omission of tritium oxide to culture vessels and gas wash water.

2.3 Preliminary Studies - Culture Characterization (Study A)

In initial studies algal growth characteristics in batch cultures were determined and procedures to be used in the measurement of tritium were examined. Stage of culture growth was followed by removing 4 mL samples from the culture vessels at one to two day intervals for determination of optical density.

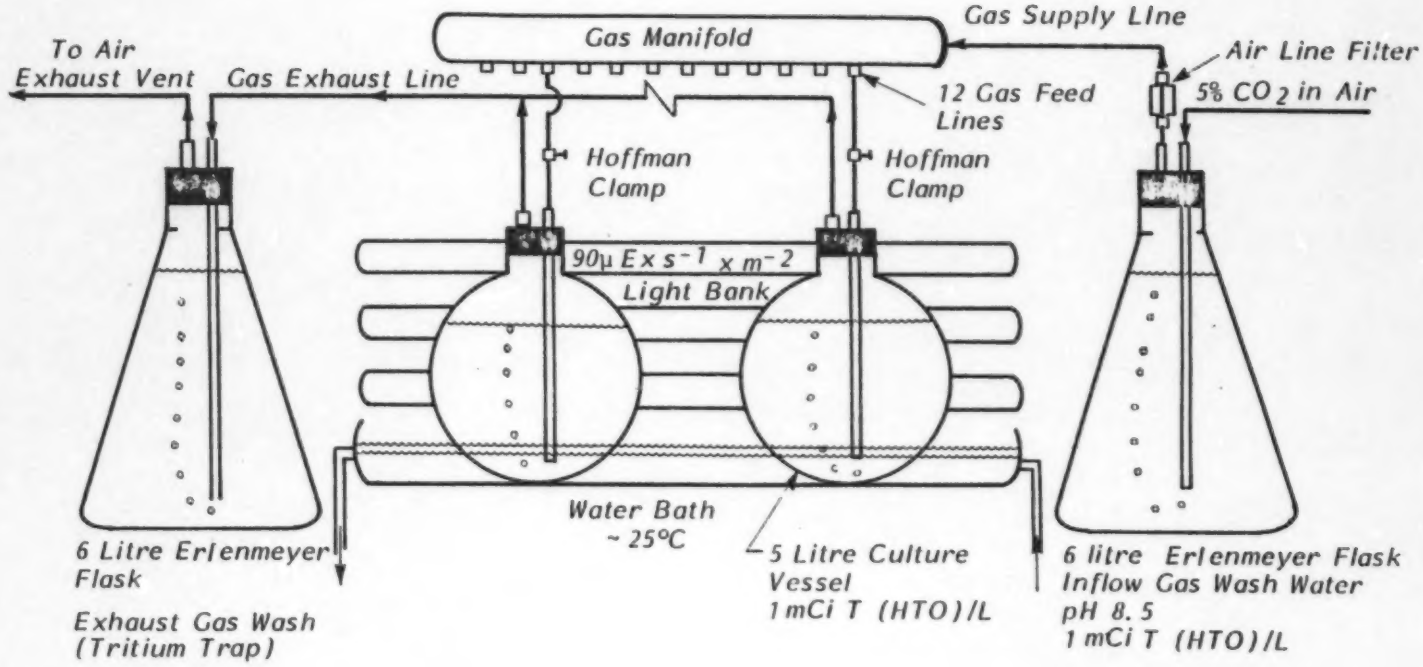

FIGURE 1
ALGAL CULTURE APPARATUS SHOWING
TWO OF TWELVE CULTURE VESSELS

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These measurements were related to cell densities to verify that optical density values were linearly related to population size. Measurements of organically bound tritium were related to dry weights of algal material combusted, and then were expressed on a per gram hydrogen basis. Tritium oxide content of culture medium and algal cells were also expressed on a per gram hydrogen basis.

Six batch cultures were incubated six to eight days by which time mid-exponential growth had been reached. Three of these cultures were serially diluted (with Beijerinck's medium) giving algal concentrations of 100% (initial undiluted culture), 50%, 25%, 12.5% and 6.25%. From each dilution 4 mL samples were taken for determination of optical density (OD). Percent transmittance (685 nm) data were converted to Log_2 OD + 10 values to provide cell doubling rates (Sorokin 1975). Samples were fixed with 0.05 mL Utermohls' solution and cell density estimates were obtained using a haemocytometer by enumerating 200-300 cells (4 replicates) under a magnification of 200 times (Guillard 1975). Algal material remaining in the dilutions was settled in complete darkness, the supernatant decanted, and the algal material further concentrated by centrifugation at 3500 *g* for 10 min. Recovered algal pellets were transferred using a minimal amount of water (10 mL) to preweighed aluminum drying dishes, covered with a layer of perforated foil, and oven dried at 105°C until constant weight was attained. Following drying, the organically bound tritium (OBT) content of undiluted samples was determined. The dilution series was used to determine areas of linearity between actual biomass and measures of biomass such as optical density, cell density and dry weight. Relationships between various parameters were also determined. The remaining three cultures were used for determination of tissue-free water tritium (TFWT) and OBT (Section 2.3.1). This procedure was repeated for six cultures grown to stationary growth phase over a 14-day period.

Non-tritiated batch cultures were grown for analysis of hydrogen content of the algal cells. Exponential and stationary cultures were centrifuged and the pellets were divided such that five levels of cell volumes were represented. Oven dried algal material was submitted to the Analytical Services Section and analyzed for hydrogen by gravimetry following sample combustion.

Initial and final weights (allowing for withdrawals during incubation) of culture vessels and contents taken during preliminary studies indicated that the change in culture volume following incubation was generally less than 1%. Chronic (steady state) medium tritium concentrations were maintained within the culture vessels for periods of up to 37 days as determined from 3 x 0.5 mL samples taken at the beginning and termination of

incubation. There was no discernible difference in tritium oxide content of algal culture suspensions or in cell-free medium (filtrates) following removal of cells with 0.45 µm Millipore filters.

2.3.1 Tritium Analyses

The three cultures grown to exponential growth phase and three to stationary growth phase were each centrifuged and the pellets from each culture were divided in half for analysis of TFWT and OBT.

2.3.1.1 Tissue-Free Water Tritium (TFWT)

The algal pellet for TFWT analysis was transferred with 10 mL distilled water into 500 mL round bottom Pyrex distillation flasks. Algal TFWT was extracted with 50 mL of an azeotropic toluene and water mixture (bp = 85°C), a modification of the procedure described by Moghissi *et al.* (1973; Ontario Hydro Research Division Report 83-33-K). Triplicate 0.5 mL aliquots of distillate were combined with 14.5 mL PCS II scintillation cocktail and samples counted for tritium activity in a Searle Mk III Liquid Scintillation Counter.

2.3.1.2 Organically Bound Tritium (OBT)

The concentrated algal material for OBT analysis was washed with tritium-free medium and recentrifuged, the procedure being repeated three times. Algal material was then oven dried and a portion of the dried material, generally 0.3 g or less, was combusted using a Packard Tri-Carb Oxidizer (Model C306). Care was taken during oxidation of samples to run a blank between samples or to process high activity samples following low activity samples, since some residual tritium activity (0.1%) was carried over in successive burns (Appendix 1). Following oxidation, combustion water was collected, mixed with Monophase 40 scintillation cocktail, and counted in a Packard Tri-Carb 300 Scintillation Counter to determine tritium content. Triplicate unwashed algal samples were also analyzed for OBT following dry weight determinations for the undiluted samples ($n = 3$) described earlier. No significant difference (t-test; $p < 0.05$) in OBT content of washed and unwashed algal material was apparent for either exponential or stationary cultures (Appendix 2) and thus the washing procedure was discontinued in subsequent analysis.

2.4 Incorporation of Tritium Oxide by Algae and Specific Activity Relationships (Study B)

Uptake of tritium oxide in relation to growth of the batch cultures was determined. Individual cultures were sequentially removed from the incubation apparatus at six points in time which spanned early exponential to stationary growth phases (Log_2 OD + 10 = 4.5, 6.0, 7.5, 8.5, 9.5 and 10.5). Replication was obtained by repeating the procedure four times. Cells were concentrated by centrifugation. Half of the packed cell volume (pellet) was dried and up to 0.3 g dry weight of algal material was combusted for determination of OBT. The remaining half of the pellet was azeotropically distilled for determination of TFWT. Initial medium tritium (HTO) levels were determined by removing triplicate 0.5 mL aliquots for scintillation counting immediately following inoculation of the cultures.

Controls consisting of non-tritiated batch cultures, harvested at the six stages of growth described above (in duplicate) were also analyzed for tritium. Background activity levels (mean ± 1 SD) for medium-T (37 ± 7 DPM/0.5 mL; n = 36), TFWT (42 ± 10 DPM/0.5 mL; n = 11) and OBT (80 ± 42 DPM per burn; n = 9) were, in all cases, several orders of magnitude less than values obtained with tritiated cultures and were well within the variation observed in replicate counts for tritiated samples. For example medium-T counts for 0.5 mL samples of tritiated medium were of the order of 480 000 ± 25 000 DPM/0.5 mL. Tritiated sample counts were, therefore, not corrected for background activity.

For experimental batch cultures, medium tritium and tissue-free water tritium values were expressed as activity (DPM) per gram hydrogen, given that 1 mL of water is equivalent to 0.111685 g H. Therefore at 20°C

$$\text{DPM/g H} = \text{DPM/mL } H_2O \times 8.954 \qquad (1)$$

Tritium activity (DPM) of combusted samples (OBT) was related to dry weight of material burned and then was converted to tritium activity per gram hydrogen

$$\text{DPM/gH} = \text{DPM/g dry wt} \times \frac{100}{\% \text{ H Content}} \qquad (2)$$

This enabled the determination of OBT to medium-T specific activity relationships.

The incorporation of tritium oxide into organic matter was described by the exponential relationship

$$R_w = R_\infty (1 - e^{-kw}) \quad (3)$$

where

R_w = the specific activity ratio of tritium in the organic fraction at a particular stage of growth (defined by dry weight) in batch culture

R_∞ = the steady state (asymptotic) value for the specific activity ratio, reached when dw/dt = 0

w = algal biomass expressed as dry weight (g)

k = initial uptake rate constant at the time cultures were exposed to tritium oxide, expressed as g^{-1}

A non-linear least squares procedure (NLIN, Statistical Analysts Systems, SAS Institute, Gary, NC) was used to fit experimental results to the model. Parameter estimates for R_∞, k and associated variances were determined by an iterative technique. It should be noted that derived 95% confidence intervals are only approximate since the model is non-linear in its parameters.

2.5 Loss of Organically Bound Tritium from Algae Transferred to Tritium-Free Medium (Study C)

Batch algal cultures grown in tritiated medium were transferred during exponential (LOG_2 OD + 10 = 7.7) and stationary (Log_2 OD + 10 = 10.8 to 11.0) growth phases to non-tritiated medium, and the time course (six points) of tritium loss from OBT was followed. The media used for the resuspensions were cell-free filtrates (0.45 filters) from non-tritiated batch cultures which had attained the same stage of growth as the tritiated cultures. This was done to ensure that exponential and stationary growth conditions following transfer remained unchanged.

When tritiated cultures had reached the appropriate growth phase (six exponential cultures, six stationary cultures) cells were harvested by centrifugation. Half of the packed cell volume (pellet) derived from the 5 L cultures was dried and up to 0.3 g of this material was combusted for determination of initial OBT content of the cells, prior to resuspension. The remaining cells in the pellet were washed with 100 mL non-tritiated medium from the same growth phase to remove as much cellular and interstitial HTO as possible, recentrifuged, and then were resuspended in 2.5 L of non-tritiated medium. The resuspended cultures in exponential growth were removed one at a time for OBT analysis at Log_2 OD + 10 values ranging from 7.6 to 10.9. Resuspended stationary cultures were sequentially removed for

OBT analysis at times following reinoculation of 16 h, 1, 2, 3, 6 and 9 days, which approximated the harvesting of reinoculated exponential cultures. Final medium-T samples (3 x 0.5 mL) were removed at the time resuspended cultures were harvested. Loss of OBT was determined as a percentage of the initial OBT level at the time of resuspension

$$\% \text{ Loss} = \frac{\text{Initial OBT} - \text{OBT at Time t}}{\text{Initial OBT}} \times 100 \qquad (4)$$

after correction for organic matter synthesized between the time of culture resuspension and culture harvesting using differences in dry weight.

3.0 RESULTS

3.1 Batch Culture Characterization (Study A)

3.1.1 Growth of Cultures

Chlorella pyrenoidosa grown in 5 L batch cultures entered exponential growth (a doubling in cell density per unit time) by the third day following inoculation. Exponential growth continued until approximately the eight day, after which the rate of growth gradually declined with cultures entering stationary growth (no net increase in cell density) after approximately 14 days (Figure 2).

During exponential growth, on days 3 to 8, average doubling time for the algal cells was slightly less than one day although the values ranged from 0.5 to 1.4 days. Variation in growth of batch cultures, as depicted in Figure 2 (data from all studies presented), was probably related to several factors. Size of the inoculum used may have differed, optical densities were not determined at precise 24 h intervals and location of the culture vessels within the incubation apparatus may have resulted in somewhat different growth rates. In subsequent studies, however, optical densities were used to determine stage of growth in individual cultures prior to measurement of tritium in algal cells. Exponential and stationary cultures were used for experimentation when optical densities (Log_2 OD + 10) had attained values of approximately 7.5 and >10.5, which corresponded with approximately the fifth and fourteenth days of incubation, respectively.

3.1.2 Growth Parameters

Optical density was an appropriate measure of culture growth (Figure 3) being related to cell density (CD) as represented by the equation

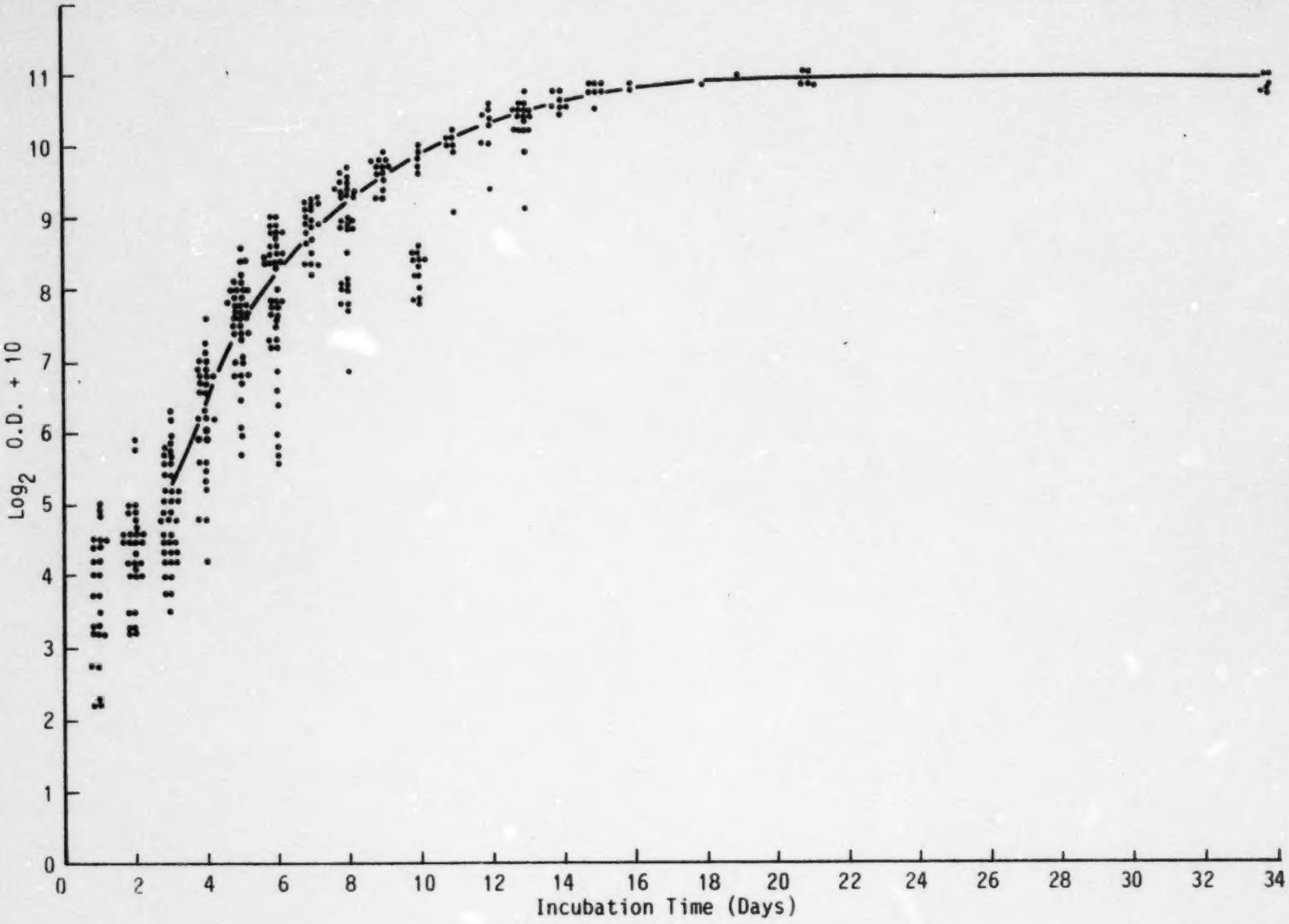

FIGURE 2

GROWTH OF CHLORELLA PYRENOIDOSA IN BATCH CULTURE AS MEASURED BY OPTICAL DENSITY (LOG_2 O.D.+10). THE LINE REPRESENTS AN APPROXIMATION OF THE GROWTH CURVE FOR A TYPICAL CULTURE

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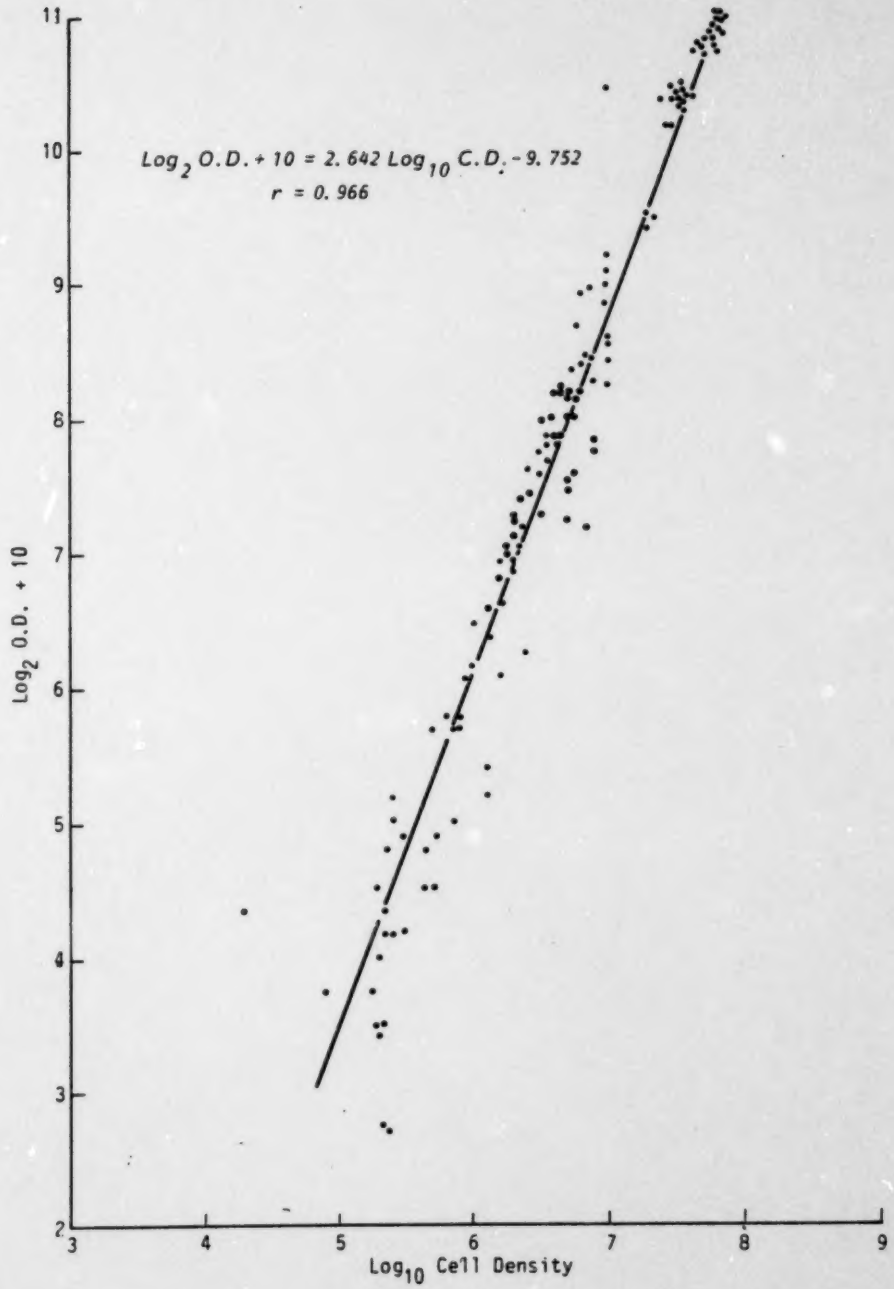

FIGURE 3

RELATIONSHIP BETWEEN OPTICAL DENSITY (LOG_2 O.D. + 10) AND POPULATION DENSITY (LOG_{10} CELLS.mL^{-1}) FOR CHLORELLA PYRENOIDOSA GROWN IN BATCH CULTURE

$$\text{Log}_2 \text{ OD} + 10 = 2.642 \text{ Log}_{10} \text{ CD} - 9.752; \; r = 0.966 \qquad (5)$$

Cell densities in cultures grown to Log_2 OD + 10 levels of 7.5 and >10.5 were approximately 3.39×10^6 and $>46.3 \times 10^6$ cells/mL, respectively.

In serially diluted cultures, in which relative population densities were 100%, 50%, 25%, 12.5% and 6.25% a linear relationship was generally found to exist between measures of growth and dilutions. Optical densities were linearly related to dilutions for Log_2 OD + 10 values between 5.0 and 10.5. At values >10.5 the relationship was obscured because of the extreme reduction in transmittance (<4%). Cell densities were linearly related to the serial dilutions and algal weights were linearly related to dilutions except in samples representing 6.25% of the initial algal concentration.

Inter-relationships between growth parameters established from the dilution series were used to estimate algal yield and population density at the time cultures were harvested (Figure 3). The relationship between algal dry weight (DW) and optical density, based on 22 data sets, was of particular interest for determining availability of material for OBT analysis:

$$\text{Log}_{10} \text{ DW} = 0.378 \; (\text{Log}_2 \text{ OD} + 10) - 3.67; \quad r = 0.96. \qquad (6)$$

3.1.3 Hydrogen Content

Hydrogen content of algal cells was determined for exponential and stationary growth phase cultures. The range in hydrogen content for 10 analyses on samples of different dry weights was 6.03% to 7.66% with no apparent relationship to the amount of material analyzed. The mean ± 1 SD for five determinations was 6.74 ± 0.60 for exponential cultures and 7.16 ± 0.25 for stationary cultures. Since no significant difference (t-test; $p > 0.05$) existed between exponential and stationary cells, the overall mean hydrogen content of 7% was assumed to be representative for all algal material for which OBT was determined. This was close to the reported values of 6.92% for *Chlamydomonas reinhardi* (Rosenthal and Stewart 1971) and 6.0% for *Scenedesmus quadricauda* (Strack *et al.* 1979).

3.2 Incorporation of Tritium Oxide by Algae and Specific Activity Relationships (Study B)

The amount of tritium incorporated into the organically bound component (OBT) of algal cells, expressed as the specific activity ratio of OBT to medium-T at first increased with increasing biomass and then approached an asymptotic value after approximately seven cell doublings (Figure 4). This was particularly

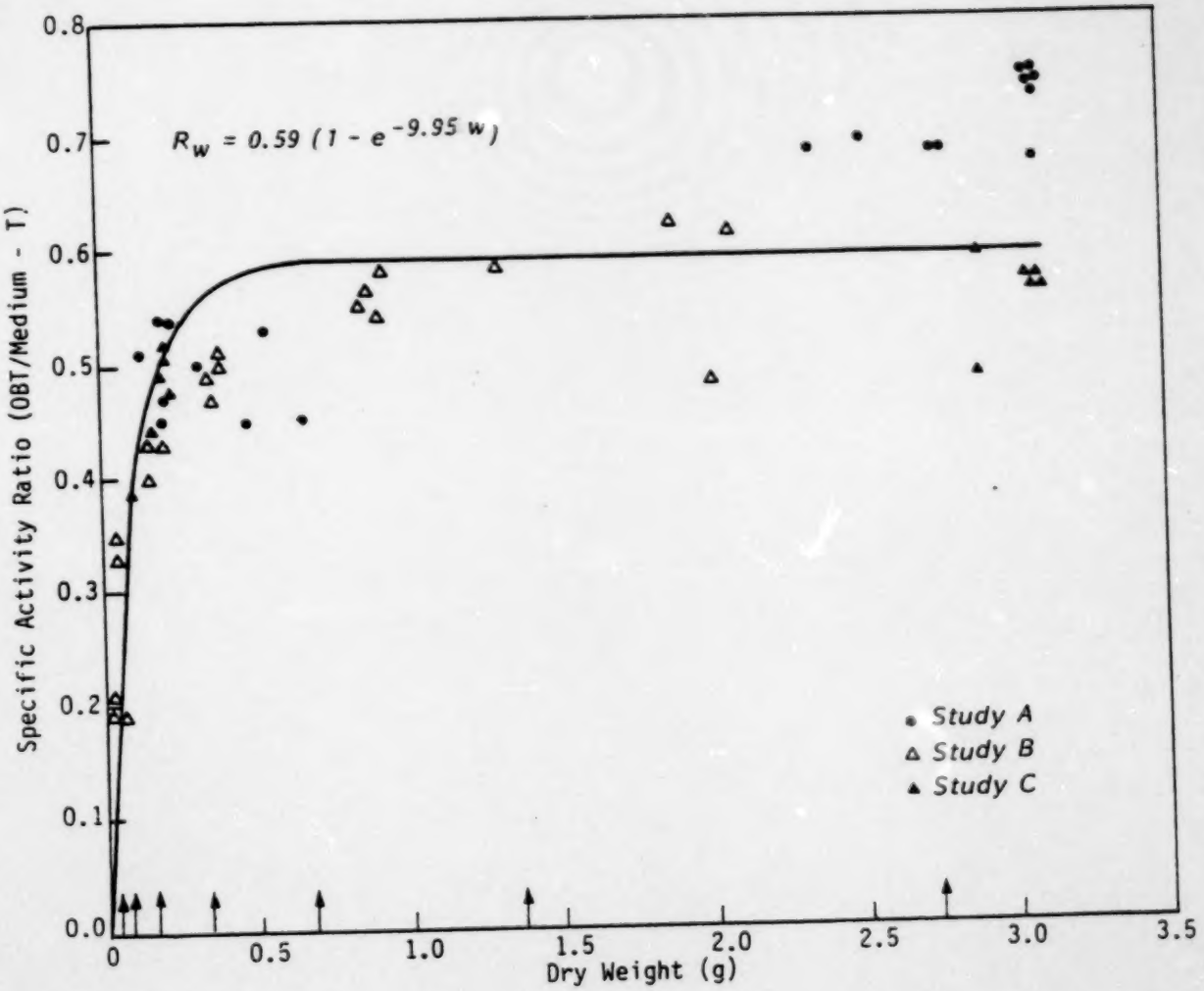

FIGURE 4

INCORPORATION OF TRITIUM OXIDE (HTO) INTO CHLORELLA PYRENOIDOSA EXPRESSED AS THE RATIO OF TRITIUM IN THE ORGANICALLY BOUND FRACTION (OBT) TO TRITIUM IN MEDIUM WATER. ARROWS ABOVE ABSCISSA INDICATE SUCCESSIVE CELL DOUBLINGS

evident in study B with OBT being measured from early exponential to stationary growth phases. The specific activity ratio increased from 0.2 to slightly more than 0.6 during this period.

Preliminary experiments (study A) had also indicated that OBT levels in stationary cultures were consistently higher than those in algae from mid-exponential growth phase. In later experiments (study C), however, the higher OBT content of stationary cultures as compared to exponential cultures was not as pronounced. Also, the specific activity ratios of stationary cultures from study C were consistently lower (range 0.48 to 0.59) than those found during preliminary studies (range 0.68 to 0.75) as shown in Figure 4. The reason for this difference in OBT content was unknown.

The exponential uptake model, used to define the relationship between specific activity and algal dry weight, was based on the 52 available data points from the three experimental sets (Appendix 4). The relationship derived for *C. pyrenoidosa*, grown in 5 L batch culture, was

$$R_w = 0.59\ (1 - e^{-9.95w}) \qquad (7)$$

where R_w represents the specific activity ratio at a particular stage of growth, measured as dry weight (w) in grams. The steady state (asymptotic) specific activity ratio was 0.59 with lower and upper 95% confidence intervals of 0.56 and 0.62, respectively. Incorporation of tritium into organic molecules therefore proceeds at approximately 59% of the rate of hydrogen incorporation. The initial uptake rate constant, at the time the algae were first exposed to HTO was $9.95.g^{-1}$ with 95% confidence intervals of 7.22 and $12.68.g^{-1}$. The rate of uptake steadily declined as the difference in the tritium to hydrogen ratio between the medium and organic molecules declined, until steady state was reached.

The tissue-free water tritium content (TFWT) of the algal cells was measured during different stages of culture growth (study B). Results varied considerably with specific activity ratios (TFWT/medium-T) ranging between 0.5 and 1.6. To measure TFWT, the supernatant following centrifugation was decanted and tritium content of the pellet (= packed cell volume) was determined following azeotropic distillation. It was not possible, however, to decant all medium tritium or to remove interstitial water present in the pellet. In subsequent determinations the amount of medium remaining in the centrifuge tubes was found to vary between 0.3 and 0.8 mL. This resulted in a substantial error to the measurements, particularly when packed algal cell

volume was less than 3 mL. Specific activity ratios, after correction for the presence of medium-T (0.6 mL assumed) were close to unity (range 0.99 to 1.18) for samples with a packed cell volume of 4 to 6 mL (Figure 5). These determinations indicated that tritium oxide concentrations within and outside the cells were close to equilibrium.

3.3 Loss of Organically Bound Tritium from Algae Transferred to Tritium-Free Medium (Study C)

Growth patterns for batch cultures of *Chlorella pyrenoidosa* were maintained following transfer of algae from tritiated media to tritium-free media. After resuspension a progressive decrease in the OBT content of the algae occurred over time (Table 1). This decrease could be attributed to two processes. Most of the decrease resulted from the synthesis of non-tritiated organic matter leading to a substantial "dilution" of the OBT present at the time of resuspension. Algae from exponential cultures grown to stationary growth phase had undergone more than three cell doublings and biomass (dry weight) at the time of resuspension represented 11.6% of the biomass at the time of harvesting.

The second component leading to a decrease in OBT concentration was the loss of tritium by cell degradation or by hydrogen-tritium exchange reactions. This loss accounted for an average decrease in OBT content of 4.4% (range for six cultures 0.7 to 9.5%) for algal material transferred during mid-exponential growth. The rates of tritium loss from the organically bound fraction were linearly related to change in dry weight (logarithmic transformation of data) between the exponential and stationary growth phases (Figure 6). Analysis of the data indicated that the loss of tritium (<5%) differed significantly ($p > 0.001$) from the theoretical OBT content expected to result solely from dilution with newly synthesized organic matter (Appendices 5,6).

Loss of previously formed organic matter by algae which were transferred during stationary growth phase averaged 13.4% (range for six cultures 5.8 to 18.6%) although the period of time spent in the tritium-free media was comparable to the exponential cultures (Table 1). There was, however, no apparent trend in the percentage of tritium lost from previously formed organic matter with respect to age or biomass (dry weight) of the cultures (Figure 6).

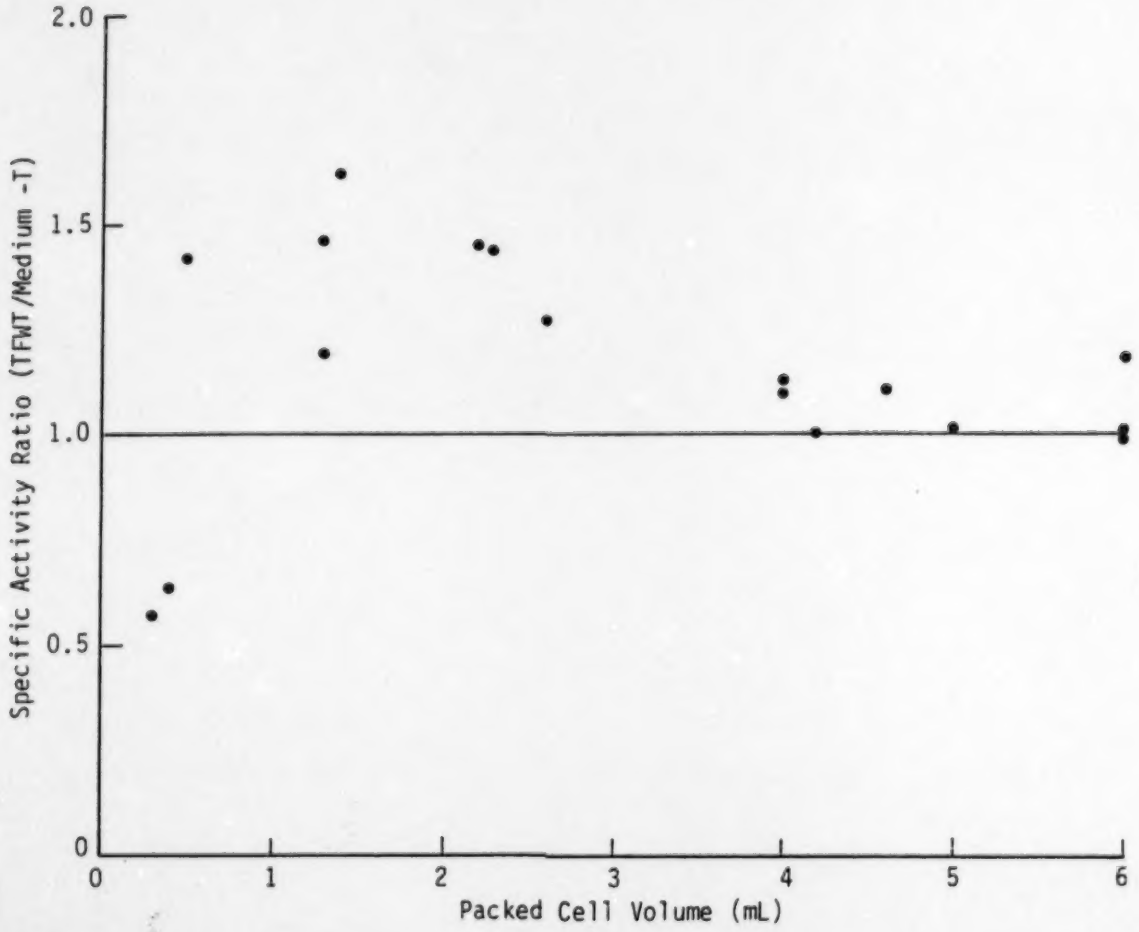

FIGURE 5

RELATIONSHIP BETWEEN TISSUE-FREE WATER TRITIUM (TFWT) SPECIFIC ACTIVITY AND THE VOLUME OF ALGAL MATERIAL ANALYZED. THE LINE SHOWN IN THE FIGURE REPRESENTS THE EXPECTED TFWT TO MEDIUM-T RATIO

TABLE 1

Loss of Organically Bound Tritium (OBT) Following the Resuspension of Algae into Tritium-Free Medium

Stage of Growth at Time of Resuspension	Incubation Period (Days) Following Resuspension (t)	Initial Dry Weight As a Per Cent of Final Dry Weight at time t	Final OBT Content As a Per Cent of Initial OBT Content at time t	Per Cent Loss of OBT
Exponential	0.67	75.5	74.8	0.7
	1	66.7	61.1	5.6
	2	48.1	38.6	9.5
	3	37.8	33.4	4.4
	7	18.0	15.0	3.0
	10	11.6	8.4	3.2
Stationary	0.67	98.9	84.9	14.0
	1	99.8	81.7	18.1
	2	91.4	78.0	13.4
	3	85.7	79.9	5.8
	6	65.8	47.2	18.6
	9	62.4	52.2	10.2

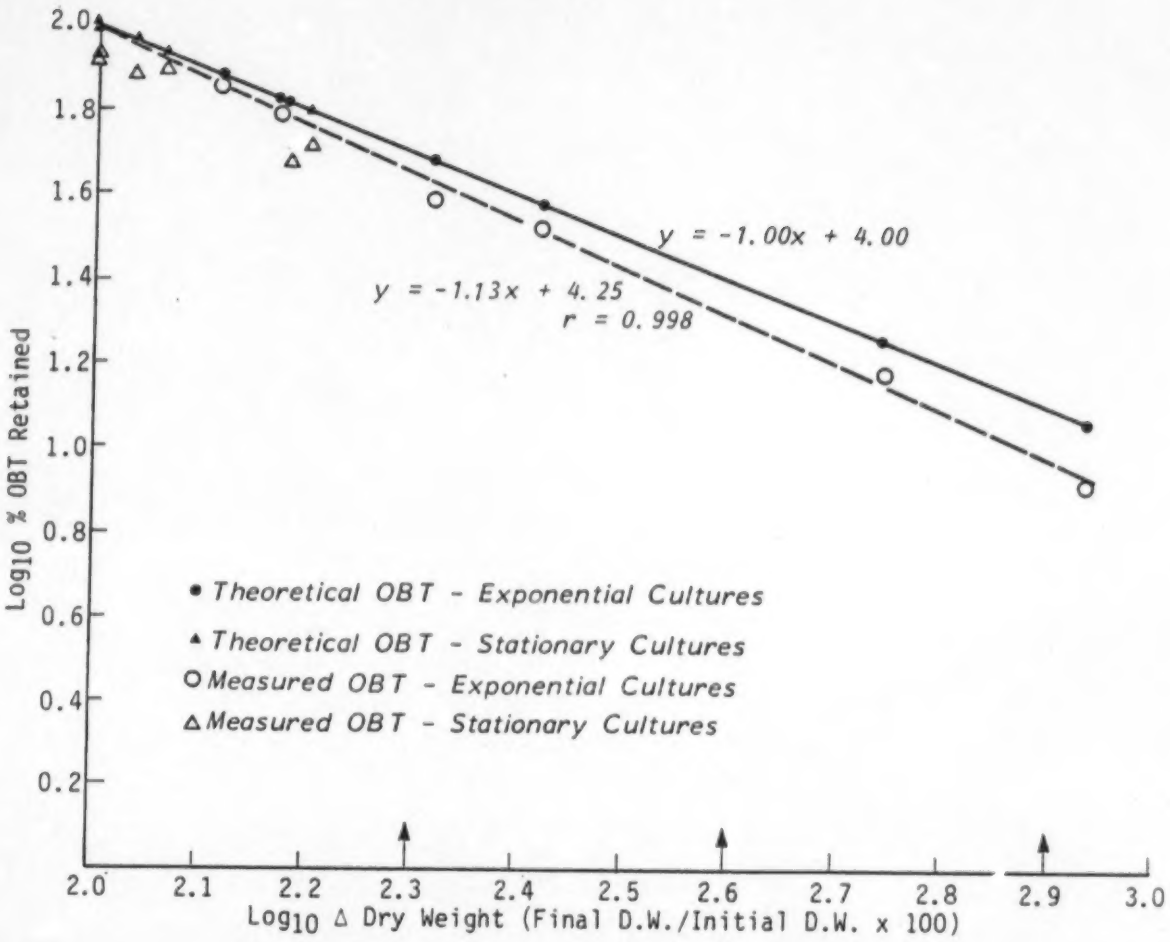

FIGURE 6

DECLINE IN ORGANICALLY BOUND TRITIUM (OBT) UPON TRANSFER OF CHLORELLA PYRENOIDOSA TO TRITIUM-FREE MEDIA. DILUTION OF THE OBT POOL BY NEWLY SYNTHESIZED ORGANIC MATTER IS SHOWN BY SOLID SYMBOLS, SOLID LINE. LOSS OF PREVIOUSLY FORMED OBT IS SHOWN FOR CULTURES INOCULATED WITH ALGAE FROM EXPONENTIAL GROWTH PHASE (OPEN CIRCLES, BROKEN LINE). ARROWS ABOVE ABSCISSA INDICATE SUCCESSIVE CELL DOUBLINGS.

Tritium oxide content of the resuspension media was measured at the time of cell harvesting (Table 2). Levels of medium HTO averaged 1500 DPM/g H (range 800 to 3700) in cultures transferred during exponential growth stage and 14 600 DPM/g H (range 9200 to 19 600) in cultures transferred during stationary growth. In both sets of cultures there was no apparent relationship of HTO concentration to length of the growth period in resuspension media.

Although the actual amount of tritium in the organically bound fraction had decreased over time (Figure 6), its detection in the culture media as HTO was obscured by the relatively high levels of HTO aleady present. This HTO was considered to have been present within the algae as TFWT upon transfer to the tritium-free resuspension cultures, indicating incomplete removal of HTO by repeated washings and centrifugation. Media tritium concentrations from exponential and stationary resuspension cultures were generally (10 of 12 values) comparable when expressed per unit dry weight of algal material initially introduced into the resuspension media (Table 2).

4.0 DISCUSSION

The behavior of tritium present as tritiated water (HTO) in the aquatic environment was modelled in the laboratory using the unicellular alga *Chlorella pyrenoidosa* as a representative primary producer. The concentration of tritium used, approximately 1 mCi/L, was much lower than the level expected to impede growth through radiological effects (about 20 Ci/L; Rambeck and Bassham 1973). Since tritiated water has similar chemical properties to natural water (H_2O), tritium concentration could be expected to be reduced through dilution in the aquatic milieu (Elwood 1971). Photosynthesis by aquatic plants and algae, however, represents a major source of entry for tritium into organic matter with the potential for subsequent transfer of tritium through consumer food chains.

In batch cultures of *C. pyrenoidosa* the intra- and extracellular concentrations of tritiated water were in equilibrium. Similarly, specific activity ratios of TFWT/medium-T in algae which are close to unity have been reported by others (Rosenthal and Stewart 1971, Kirchmann *et al.* 1979, Harrison and Koranda 1973). Equilibration of tritium into TFWT occurs rapidly in algae, generally within minutes.

Tritiated water was discriminated against in the biosynthetic reactions of *C. pyrenoidosa*. The steady state specific activity ratio observed for organically bound material (OBT/medium-T) was 0.59. Kanazawa *et al.* (1972) and Wienberger and Porter (1953) reported organically bound specific activity ratios of about 0.7

TABLE 2

Concentration of HTO in the Medium of Resuspension Cultures at the Time of Harvest[1]

Stage of Growth At Time Of Resuspension	Incubation Period (Days) Following Resuspension	HTO Concentration in Medium (DPM/g H x 10^{-3})	HTO Concentration Per Unit Algal Material Resuspended (DPM/g H/g DW x 10^{-3})
Exponential	0.67	3.7	25.0
	1	1.3	5.3
	2	1.1	4.8
	3	0.9	4.0
	7	0.8	4.5
	10	1.2	5.2
Stationary	0.67	19.6	10.7
	1	13.3	7.0
	2	13.3	6.1
	3	9.2	4.5
	6	13.3	7.3
	9	19.1	8.6

[1] Average HTO concentration in culture media prior to resuspension in tritium-free media was 19.1 x 10^{6} DPM/gH.

and 0.5, respectively, for *C. pyrenoidosa* grown in tritiated media. Specific activities for individual compounds have been reported to range from approximately 0.45 to 1.16. Little discrimination (R = 1) was apparent for intermediates of the tricarboxylic acid cycle and their derivatives while ratios of about 0.5 to 0.7 existed for other metabolites (Kanazawa *et al.* 1972, Rambeck and Bassham 1973). Differences in growth conditions could lead to a shift in biosynthetic reactions, thereby altering OBT specific activity ratios for whole organisms as noted by Kanazawa *et al.* (1972). Incorporation of tritium into intermediates of the tricarboxylic acid cycle would be greatest during exponential growth since a large portion of energy is directed to synthesis of organic matter. In the stationary growth phase, energy is used primarily for maintenance metabolism. Specific activity ratios (OBT/medium-T) for both marine and freshwater algae have been found to be relatively consistent, however, ranging from approximately 0.5 to 0.80 (Ibid, Rosenthall and Stewart 1971, Strack *et al.* 1979, Strack 1978, Komatsu *et al.* 1981, Kirchmann *et al.* 1979). Tritium oxide present in the aquatic environment is not concentrated into the organically bound fraction of primary procedures (ie: specific activity <1) when grown under chronic exposure conditions.

Discrimination against tritium in biosynthetic reactions is attributed to the high mass of tritium relative to hydrogen and because the mass of the water molecule is relatively small (McKinley and Wetzel 1976). The amount of tritium incorporated into organic molecules is, however, related to the concentration of tritium oxide in the medium since the discrimination factor remains constant (Kirchmann *et al.* 1979, Bonotto *et al.* 1977).

Tritium incorporated into organic matter could be expected to remain bound in healthy cells for a relatively long period of time. Less than 5% of OBT present in actively growing cells of *C. pyrenoidosa* was lost after three cell doublings. Loss of tritium from organic molecules could occur through catabolic metabolism or by hydrogen-tritium exchange reactions. The small amount of tritium lost from OBT (<5%) is in agreement with results of Wienberger and Porter (1953) and Strack *et al.* (1979). The rate of tritium loss from OBT was higher at about 13% over a comparable period of time for cells in the stationary growth phase. This was comparable to the value of 10% reported by Strack (1978) for old cultures of *Scenedesmus quadricauda*. Accelerated loss of OBT among senescent cells may be related to increased autolysis and decomposition.

This relatively slow rate of tritium loss could result in specific activity levels which exceed one under dynamic environmental conditions in which a pulse of tritium oxide is dissipated quickly over time. This was demonstrated for the alga

S. quadricauda which was maintained in continuous culture and received a single injection of tritium oxide. The decline in the concentration of OBT was found to proceed at a slower rate than the elimination curve for HTO (Strack *et al.* 1979). The preferential incorporation of tritium into compounds of the tricarboxylic acid cycle indicated that once incorporated, however, tritium would be preferentially retained during oxidative reactions (Kanazawa *et al.* 1972). Selective retention of tritium in organically bound form may persist in consumer organisms which rely on the oxidative metabolism of food for energy (Ibid). Results of the present study indicate that care should be taken in interpreting specific activity relationships for organisms collected from natural aquatic environments or under condition where a decline in tritium oxide concentration has occurred. Ratios of OBT/HTO which exceed unity do not necessarily imply bioconcentration of tritium but rather may reflect persistence of tritium within organic matter.

5.0 SUMMARY AND CONCLUSIONS

1. The alga, *Chlorella pyrenoidosa*, was used to model the behavior of tritium in an aquatic primary producer. Cells were grown in 5 L batch cultures containing tritium oxide (HTO) at a concentration of approximately 1 mCi/L.

2. The specific activity of organically bound tritium to medium (HTO) tritium increased during initial culture growth but then approached an asymptotic value of 0.59 after approximately seven cell doublings. This indicated a discrimination against tritium (as HTO) in biosynthetic reactions which was comparable to specific activity ratios of 0.5 to 0.7 previously reported for *Chlorella pyrenoidosa*. No bioconcentration of tritium oxide into organic matter occurred under steady state conditions of exposure.

3. The concentrations of tissue-free water tritium (TFWT) and medium HTO appeared to be in equilibrium. However, methodological difficulties resulted in inconsistent specific activity ratios (TFWT/medium-HTO) when the amount of algal material being analyzed was small (<3 mL packed cell volume).

4. The concentration of previously formed OBT in cells transferred to media free of HTO was diluted by newly synthesized, non-tritiated, organic matter. A small but significant loss (<5%) of OBT, resulting from catabolic metabolism and/or hydrogen-tritium exchange reactions, occurred among actively growing cells which had undergone more than three cell doublings. Over a comparable period of time loss of OBT among senescent (stationary) cells was higher (average

13.4%), a probable result of increased degradative metabolism. Under dynamic conditions in which a pulse of tritium oxide to the environment is dissipated quickly over time, the concentration of tritium may persist in organic molecules and eventually exceed HTO concentrations. Specific activity of OBT/HTO in biological samples, taken from environments which are periodically exposed to elevated levels of tritium oxide, may reflect stability of tritium in the organically bound fraction rather than bioconcentration of this radiosotope.

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APPENDIX 1

OXIDATION OF ORGANIC MATTER - PACKARD TRI-CARB OXIDIZER - TRITIUM CARRY-OVER INTO SUBSEQUENT SAMPLES

Blanks - consists of 2 cups + pad - Mean ± 1 S.D. for 11 blanks run at beginning of day prior to further oxidations = 53 ± 14 DPM.

		DPM	DPM Carry-Over to Blank (-Bckg)	Carry-Over as a Per Cent of Sample (or Std) Activity
A)	Std	38 629		
	Blk #1	95	42	0.1
	Blk #2	53	-	-
	Blk #3	62	-	-
B)	Sample	465 148		
	Blk #1	378	325	0.1
	Blk #2	125	72	0.02
	Blk #3	74	21	0.005
	Blk #4	61	-	-
C)	Sample	479 627		
	Blk #1	624	571	0.1
	Blk #2	143	90	0.02
	Blk #3	81	28	0.006
	Blk #4	71	18	0.004

Variation in DPM's for Standards - 37 662, 38 629, 38 019, 36 056] Approximate % Variation = 4%

APPENDIX 2

COMPARISON OF ORGANICALLY BOUND TRITIUM ANALYSES FOR WASHED VERSUS UNWASHED ALGAL MATERIAL

a) Exponential Cultures

OBT Activity (µCi/g dry wt)			
Washed	Log_2OD+10	Unwashed	Log_2OD+10
0.30	7.28		
0.32	7.85	0.28	8.19
0.27	9.23		
0.27	8.85	0.31	8.99
0.29	8.37		
0.32	7.85	0.27	7.83
*Mean ± 1 S.D. 0.30 ± 0.02		0.29 ± 0.02	

b) Stationary Cultures

OBT Activity (µCi/g dry wt)			
Washed	Log_2OD+10	Unwashed+	Log_2OD+10
0.41	10.76	0.43	11.0
2.22?		0.40	11.0
0.41	10.87	0.44	11.0
0.40	10.87	0.45	11.0
No Sample		0.44	11.0
0.41	10.68	0.44	11.0
*Mean ± 1 S.D. 0.41 ± 0.01 (Value of 2.22 not included)		0.43 ± 0.02	

* No significant difference between washed and unwashed treatments (t-test; $p \leq 0.05$).

\+ Slightly higher OBT activity in unwashed stationary culture is probably related to a more advanced stage of growth as indicated by optical density values.

APPENDIX 3

Incorporation of Tritium Oxide (HTO) by *Chlorella Pyrenoidosa*

Culture	$Log_2OD + 10$	Organically Bound Tritium DPM/gH x 10^{-6}	Specific Act Ratio+ (OBT/Medium-T)	Tissue Free Water Tritium DPM/gH x 10^{-6}	Specific Act Ratio+ (TFWT/Medium-T)
Study A - Preliminary Studies					
13	8.19	8.76	0.47		
23	8.99	9.94	0.53		
33	7.83	8.50	0.45		
101	11.00	13.76	0.73		
		12.59	0.67		
111	11.00	14.15	0.75		
		13.86	0.74		
121	11.00	13.94	0.74		
		14.07	0.75		
12	7.28	9.49	0.51		
22	7.85	10.17	0.54		
32	9.23	8.43	0.45		
42	8.85	8.54	0.45		
52	8.37	9.32	0.50		
62	7.85	10.08	0.54		
71	10.76	13.04	0.69		
81	10.87	12.85	0.68		
		12.76	0.68		
91	10.68	12.84	0.68		
Study B - Incorporation					
A1	4.52	3.68	0.19		
A2	4.66				
A3	4.52	3.96	0.21	10.93	0.57
A4	4.66				
B1	5.96	6.69	0.35		
B2	5.96	6.31	0.33		
B3	6.07			27.07	1.42
B4	6.01	3.71	0.19	12.16	0.64
C1	7.53	8.28	0.43		
C2	7.58	7.63	0.40	27.86	1.46
C3	7.82	8.13	0.43	31.00	1.62
C4	7.58	8.21	0.43	22.74	1.19
D1	8.63	9.71	0.51		
D2	8.51	9.38	0.49	27.76	1.45
D3	8.55	8.98	0.47	27.52	1.44
D4	8.63	9.58	0.50	24.19	1.27
E1	9.62	11.05	0.58	21.57	1.13
E2	9.55	10.62	0.56	20.94	1.10
E3	9.51	10.53	0.55	19.09	1.00
E4	9.60	10.36	0.54	19.47	1.02
F1	10.43	11.85	0.62	22.57	1.18
F2	10.48	9.19	0.48	18.93	0.99
F3	10.54	11.70	0.61	18.86	0.99
F4	10.00	11.13	0.58	21.24	1.11
Study C - Loss (Initial OBT at the time of resuspension)					
EA	7.10	7.41	0.39		
EB	7.83	10.00	0.52		
EC	7.88	9.03	0.47		
ED	7.88	9.42	0.49		
EE	7.63	8.38	0.44		
EF	7.88	9.74	0.51		
SA	11.00	10.83	0.57		
SB	11.00	10.74	0.56		
SC	11.00	10.61	0.56		
SD	10.88	9.24	0.48		
SE	10.87	11.19	0.59		
SF	11.00	10.70	0.56		

+ Specific Activity Ratios Determined Using Medium-T Values of 0.94 $mCi \cdot L^{-1}$ or 18.80×10^4 $DPM \cdot gH^{-1}$ for Study A Results and 0.96 $mCi \cdot L^{-1}$ or 19.10×10^4 $DPM \cdot gH^{-1}$ for Studies B and C.

APPENDIX 4

Incorporation of Tritium Oxide into the Organically Bound Fraction. Non-Linear Least Squares Summary Statistics Describing the Relationship* Between Specific Activity Ratio (OBT/medium-T) and Algal Dry Weight (g).

Source	Degrees of Freedom	Sum of Squares	Mean Square
Regression	2	14.670797	7.335399
Residual	50	0.379403	0.007588
Uncorrected Total	52	15.050200	
Corrected Total	51	0.885208	

Parameter	Estimate	Standard Error	95% Confidence Intervals Lower	Upper
Asymptote (R_∞)	0.5902	0.0159	0.5582	0.6222
Rate Constant (k)	9.9515	1.3585	7.2229	12.6801

*$R_w = R_\infty (1 - e^{-kw})$;

R_w = specific activity ratio at a stage of growth defined by dry weight.
R_∞ = steady state (asymptotic) specific activity ratio
w = algal dry weight (g)
k = uptake rate constant upon introduction of HTO.

APPENDIX 5

Results of Study C - Loss of Organically Bound Tritium

Culture	$Log_2OD + 10$ At Resuspension	$Log_2OD + 10$ At Harvest	Dry Weight (g) At Resuspension	Dry Weight (g) At Harvest	No days Resuspended	OBT Content (DPM/gH x 10^{-6}) At Resuspension	OBT Content (DPM/gH x 10^{-6}) At Harvest
EA	7.05	7.58	0.148	0.196	0.67	7.41	5.55
EB	7.83	8.47	0.244	0.366	1	10.00	6.11
EC	7.88	8.97	0.230	0.478	2	9.03	3.49
ED	7.88	9.44	0.228	0.603	3	9.42	3.15
EE	7.63	10.13	0.178	0.991	7	8.38	1.26
EF	7.88	10.87	0.232	1.992	10	9.74	0.82
SA	11.00	11.00	1.837	1.858	0.67	10.83	9.19
SB	11.00	11.00	1.905	1.909	1	10.74	8.76
SC	11.00	11.00	2.184	2.389	2	10.61	8.28
SD	10.88	11.00	2.064	2.409	3	9.24	7.29
SE	10.87	11.00	1.814	2.758	6	11.19	5.28
SF	11.00	11.00	2.214	3.546	9	10.70	5.59

APPENDIX 6

Decline in Organically Bound Tritium From Exponential Cultures, Comparison of Measured and Theoretical Losses

DEP VARIABLE: LOGOBT LOG % OBT RETAINED

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F VALUE	PROB>F
MODEL	1	0.893348	0.893348	1255.501	0.0001
ERROR	5	0.003557735	0.0007115471		
C TOTAL	6	0.896906			

ROOT MSE	0.026675	R-SQUARE	0.9960
DEP MEAN	1.553000	ADJ R-SQ	0.9952
C.V.	1.717633		

VARIABLE	DF	PARAMETER ESTIMATE	STANDARD ERROR	T FOR H0: PARAMETER=0	PROB > |T|	VARIABLE LABEL
INTERCEP	1	4.253777	0.076886	55.326	0.0001	INTERCEPT
LOGDW	1	-1.130843	0.031915	-35.433	0.0001	LOG FINAL DW/INITIAL DW *100

TEST: SLOPE1 NUMERATOR: .0119597 DF: 1 F VALUE: 16.8080
DENOMINATOR: 7.1E-04 DF: 5 PROB >F : 0.0094

OBS	ACTUAL	PREDICT VALUE	STD ERR PREDICT	LOWER95% MEAN	UPPER95% MEAN	LOWER95% PREDICT	UPPER95% PREDICT	RESIDUAL	STD ERR RESIDUAL	STUDENT RESIDUAL
1	2.000	1.992	0.015975	1.951	2.033	1.912	2.072	0.007910	0.021362	0.370
2	1.874	1.854	0.013186	1.820	1.888	1.778	1.931	0.019873	0.023188	0.857
3	1.786	1.793	0.012147	1.762	1.824	1.718	1.868	-.007062	0.023749	-0.297
4	1.587	1.632	0.010329	1.606	1.659	1.559	1.706	-.045482	0.024594	-1.849
5	1.524	1.515	0.010139	1.489	1.541	1.442	1.588	0.009126	0.024673	0.370
6	1.176	1.148	0.015231	1.109	1.188	1.070	1.227	0.027519	0.021899	1.257
7	0.924000	0.935883	0.020124	0.884153	0.987613	0.849989	1.022	-.011883	0.017509	-0.679

SUM OF RESIDUALS 6.71685E-15
SUM OF SQUARED RESIDUALS 0.003557735

OBS	RESIDUAL	RSTUDENT	HAT DIAG H	COV RATIO	DFFITS	DFBETAS INTERCEP	DFBETAS LOGDW
1	.0079096	0.3358	0.3587	2.3046	0.2511	0.2139	-0.1948
2	.0198725	0.8299	0.2444	1.5049	0.4720	0.3489	-0.3042
3	-.007062	-0.2684	0.2074	1.9022	-0.1373	-0.0908	0.0766
4	-.045482	-2.9425	0.1499	0.1835	-1.2357	-0.4242	0.2684
5	.0091256	0.3354	0.1445	1.7278	0.1379	0.0035	0.0146
6	.0275189	1.3588	0.3260	1.0852	0.9451	-0.6202	0.7084
7	-.011883	-0.6371	0.5692	2.9893	-0.7322	0.5801	-0.6337

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